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14. ABSTRACT

Fatty acid synthase (FAS) has shown promise as a new target for breast cancer therapy. FAS is the primary enzyme responsible for the de novo synthesis of fatty acid and is highly expressed in most common human cancers, including breast, colorectal, prostate, ovary, and lung. Moreover, high levels of FAS have been found in cancer precursor lesions of the breast, prostate, and colon. In contrast, dietary fat down-regulates FAS and fatty acid synthesis in most normal tissues. To test the effect of FAS inhibition in cancer, we have developed a novel, chemically stable, small molecule FAS inhibitor, C75, that induces apoptosis in human breast cancer cells in vitro and in vivo. C75 has shown significant anti-tumor effect against MCF-7 human breast cancer xenografts in athymic mice. In light of these data, we have demonstrated that inhibition of FAS delays or eliminates mammary cancer in the neu-N transgenic mouse mammary cancer model. Moreover, treatment reduced the expression of genes known to be involved in neu signal transduction.

15. SUBJECT TERMS
breast cancer, her2/neu, fatty acid synthase

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4. INTRODUCTION Fatty acid synthase (FAS) is the primary enzyme responsible for the de novo synthesis of fatty acid and is highly expressed in most common human cancers, including breast, colorectal, prostate, ovary and lung. Moreover, high levels of FAS have been found in cancer precursor lesions of the breast, prostate and colon. To test the effect of FAS inhibition in cancer, we have developed a novel, chemically stable small molecule FAS inhibitor, C75, the induces apoptosis in human breast cancer cells in vitro and in vivo. In light of these data, we performed a pilot/initial study of C75 treatment against the neu-N transgenic mouse mammary cancer model that significantly delayed the appearance of mammary cancer in these mice. Using this model, we studied pre-neoplastic lesion development, apoptosis, DNA synthesis and expression of FAS, neu, and the neu signaling pathway (Akt, Phospho-Akt, p21/Waf1) in neu-N mice after acute and chronic FAS inhibition. Following 8-10 weeks of C75 treatment, there was a significant reduction of both the number of mammary duct structures, their thickness and the number of budding epithelial structures. Additionally, apoptotic changes were increased, DNA synthesis was decreased, and FAS, neu, Akt, Phospho-Akt and p221/Waf1 were all decreased when compared to controls. Importantly, these effects were restricted to the breast epithelial cells that overexpress neu, and not to other normal duct structures in the skin, liver or kidney. These data indicate that C75 exhibits relatively specific action against neu overexpressed mammary epithelial cells in neu-N transgenic mice, and downregulates or inhibits key components of the neu signaling pathway (1).

5. BODY For clarity, I will provide our data in the outline of the Tasks as proposed in the Statement of Work.

Specific Aim 1. ***FAS inhibition with C75 promotes apoptosis of pre-neoplastic lesions in neu-N mice by altering the neu anti-apoptotic signaling pathway.***

We have completed the studies outlined in Aim 1: treatment, immunohistochemistry, and data analysis for the 10 week C75 treatment trials. We have found that C75 retards mammary development, reduces FAS, p21, Akt, and phosphoAkt expression in mammary ducts. In addition, Brdu incorporation was decreased and apoptosis was increased. Early morphological changes occurred after 8 weeks of treatment, with easily identifiable changes after 12 weeks of treatment. These findings are presented and discussed in the paper which was published in Oncogene 24: 39-46, 2005, and is included in the Appendix (1).

C247, an FAS inhibitor, also reduces breast duct development in transgenic *neu* mice.

Rationale: The dramatic weight loss seen with C75 precludes its use as a compound for human trials for chemoprevention. We have shown that a significant mechanism of the weight loss is due to increased fatty acid oxidation (4, 5) As part of other ongoing projects, the FAS working group has been attempting to separate the weight loss attributes of C75 from its cytotoxicity to human cancer cells. Recently, our collaborators developed an FAS inhibitor based on a thiolactomycin structure. Thiolactomycin has been shown to inhibit Type II found in plants and bacteria, but not Type I FAS found in yeast and mammals (2,3). Townsend and McFadden have modified thiolactomycin to inhibit Type I FAS. C247 is a candidate cancer compound that inhibits purified human FAS, is cytotoxic to both MCF7 and OVCAR3 ovarian cancer cells, does not affect the CPT-1 system, does not increase

fatty acid oxidation and as such, does not cause weight loss. The following table compares important attributes of C247 to C75:

Drug	Type	Human FAS IC_{50} (µg/ml)	MCF7 XTT IC_{50}(µg/ml)	OVCAR3 XTT IC_{50} (µg/ml)	CPT-1 Stimulation MCF7	Weight Loss Balb/C Lean
C247	T	4.0 ± 0.5	17.6 ± 0.1	25.3 ± 0.1	None	2% @ 60mg/kg
C75	A	Slow binder	10.7 ± 2.2	7.1 ± 3.9	300% at 20 µg/ml	15% @ 30 mg/kg

The final results and methods with the C247 is also contained in the published study.

Relevance: These data demonstrate that this effect is due to FAS inhibition not fatty acid oxidation stimulation since C247 does not stimulate fatty acid oxidation. Moreover, these findings are not unique to C75. We postulate that they should occur with any FAS inhibition of similar potency. Thus, we postulate that FAS is a potential target for breast cancer chemoprevention.

Specific Aim 2. Hypothesis: Mammary tumor prevention by C75 will not be significantly altered by dietary fat.

We have noted that the *neu* mice do not become obese on the high fat diet (60% of calories from fat, 25% protein, 20% carbohydrate) that is used for diet induced obesity in male C57B6 mice. Thus, chemoprevention / obesity studies will require another animal model. We have proposed to study chemoprevention with new FAS inhibitors in diet-induced obese rats or Zucker (*fa/fa*) rat DMBA mammary carcinogenesis model (6). This work has been proposed as part of the Breast Cancer SPORE submitted from the Johns Hopkins Oncology Center (6).

6. KEY RESEARCH ACCOMPLISHMENTS

a. C75 retards mammary development in transgenic *neu* mice with a reduction in the caliber, number and budding of breast ducts easily identified after 8 weeks (8 doses) of C75, i.p. at 30 mg/kg every week.

b. C75 reduces Akt expression in the mammary epithelium.

c. C75 reduces pAkt expression in the mammary epithelium.

d. C75 reduces cell proliferation and apoptosis in the mammary epithelium.

f. C75 does not affect the mammary ducts or lobules in wild type control mice demonstrating that only cells that overexpress *neu* are affected inhibition of FAS by C75.

e. C247, an inhibitor of FAS that does not promote fatty acid oxidation or weight loss, reduces the caliber and budding of breast ducts similar to C75.

f. Since C247 caused a similar effect to C75, weight loss is not the cause of the reduced mammary development.

g. The morphological changes of FAS inhibition on mammary epithelium were first noted following 8 weeks of C75 treatment. Established easily identifiable morphological changes were noted after 10 weeks of C75 or C247 treatment.

h. ***FAS is a potential drug target for breast cancer chemoprevention.***

7. REPORTABLE OUTCOMES

a. The effects of C75 and C247 treatment on the HER2/*neu* mouse mammary cancer model has been reported in Oncogene as noted above.

b. The transgenic mice do not become obese on the high fat diet requiring a rat model to study the interactions of obesity, mammary cancer development and FAS inhibition.

8. CONCLUSIONS

C75 treatment of *neu* transgenic mice has resulted in a dramatic reduction in mammary epithelial structures. This is reflected in reduced expression of FAS and BrdU incorporation in the duct epithelial cells indicating reduced cellular proliferation. Importantly, these effects are restricted to the breast epithelial cells which overexpress *neu*. We believe this inhibition of epithelial proliferation by C75 is directly responsible for its inhibition of tumor development in this model.

The importance of the C247 data cannot be underestimated. This demonstrates that this effect is due to FAS inhibition not fatty acid oxidation stimulation. It effectively demonstrates that weight loss is not the cause of the retarded breast development. Moreover, it suggests that any FAS inhibitor with similar potency *in vivo* should have a similar effect. Therefore, we have identified FAS as a target for breast cancer chemoprevention.

Finally, since C247 is a thiolactomycin derivative, it is likely to be rapidly excreted in the urine with a short half-life and reversible binding. All of these are attributes that would be suitable for a compound for cancer prevention that would be likely to be administered for years.

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10. APPENDIX

Alli PM, Pinn ML, Jaffee EM, McFadden JM, Kuhajda FP. Fatty acid synthase inhibitors are chemopreventive for mammary cancer in *neu*-N transgenic mice. *Oncogene* 24: 39-46, 2005.

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ORIGINAL PAPER

Fatty acid synthase inhibitors are chemopreventive for mammary cancer in *neu*-N transgenic mice

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High levels of fatty acid synthase (FAS) have been found in cancer precursor lesions of the colon, stomach, esophagus, oral cavity, prostate, and breast. Inhibition of FAS with C75 has led to a significant antitumor effect in both human breast and prostate cancer xenografts. Recently, HER2/*neu*, which has also been identified in preneoplastic breast lesions, has been shown to regulate FAS expression through the PI3K/Akt signal transduction pathway rendering them susceptible to FAS inhibition. Utilizing the *neu*-N transgenic mouse model of mammary cancer, weekly treatment of the *neu*-N mice with C75 (30 mg/kg) for 10 weeks significantly delayed tumor progression. Only 20% of the C75-treated transgenic mice developed mammary carcinoma by 220 days, compared to 50% in the vehicle control animals. Two C75-treated animals never developed mammary cancer. Analysis of mammary tissue following 10 weeks of C75 treatment revealed a significant delay in mammary maturation as manifested by a reduction of the number and caliber of mammary ducts and budding epithelial structures. Apoptotic changes were increased, DNA synthesis was decreased, and the expression of FAS, *neu*, Akt, phospho-Akt, and $p21^{waf1}$ were all decreased when compared to vehicle control and FVB/N mice. Importantly, these effects were restricted to the breast epithelial cells that overexpress *neu* not involving other normal duct structures in the skin, liver, or kidney. C247, an FAS inhibitor chemically distinct from C75, significantly delayed mammary maturation similar to C75. Thus, pharmacological inhibition of FAS affects the expression of key oncogenes involved in both cancer development and maintenance of the malignant phenotype. Moreover, these data identify FAS as a potential novel drug target for breast cancer chemoprevention.
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Keywords: fatty acid synthase; HER2; *neu*; transgenic

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Introduction

The optimum management for breast cancer is prevention. Until recently, early diagnosis was the only option for women to prevent breast cancer morbidity and mortality. However, chemoprevention trials have begun that are aimed at eliminating or significantly delaying the onset of breast cancer in women at risk. Tamoxifen trials have shown promise, reducing the risk of breast cancer by nearly 50% (Fisher *et al*., 1998). While endocrine approaches to prevention and treatment of breast cancer are encouraging, their toxicities, notably endometrial carcinoma, deep vein thrombosis, and pulmonary emboli, although confined predominantly to women over 50, preclude their widespread use in the general population (Fisher *et al*., 1998; Jordan, 2000). New classes of agents are also under investigation including aromatase inhibitors, gonadotropin-releasing hormone agonists, tyrosine kinase inhibitors, polyamine synthesis inhibitors (Fabian, 2001), and vaccines (Reilly *et al*., 2000, 2001a, b, 2002). For chemoprevention to be available to most if not all women, it must be minimally toxic as treatment will likely begin in the 30s, well before the maximal risk of cancer development, and continue for years.

We have developed a novel approach to breast cancer treatment targeting the enzyme fatty acid synthase (FAS) (Kuhajda *et al*., 2000; Pizer *et al*., 2000). FAS is responsible for the *de novo* synthesis of long-chain fatty acids through catalysing the NADPH-dependent condensation of acetyl-CoA and malonyl-CoA (Wakil, 1989). Inhibition of FAS in breast and prostate cancer cells led to apoptosis both *in vitro* and *in vivo* (Pizer *et al*., 2000, 2001; Zhou *et al*., 2003). Thus, FAS has been identified as a potential drug target for chemotherapy of established cancer.

While FAS has been shown to be expressed in many common human solid tumors (Kuhajda, 2000), FAS has also been identified in preneoplastic lesions of the colon (Rashid *et al*., 1997; Visca *et al*., 1999), prostate (Epstein *et al*., 1995; Bull *et al*., 2001), stomach (Kusakabe *et al*., 2002), esophagus (Nemoto *et al*., 2001), oral cavity (Krontiras *et al*., 1999), and breast (Milgraum *et al*., 1997; Alo *et al*., 2001). HER2/*neu*, which has also been

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identified in preneoplastic breast lesions (Xu *et al*., 2002), has been shown to upregulate FAS expression through the PI3K/Akt signaling pathway rendering cells susceptible to FAS inhibition (Kumar-Sinha *et al*., 2003). Recently, FAS inhibition has been shown to suppress HER2/*neu* overexpression in breast cancer cells suggesting an active role of FAS in cancer progression (Menendez *et al*., 2004). The appearance of high levels of FAS and *neu* expression in preneoplastic breast lesions suggest that both FAS and *neu* may be targets for cancer prevention.

In this study, we are testing the utility of FAS as target for breast cancer chemoprevention using the transgenic *neu*-N mouse model in which the females develop mammary carcinoma within 300 days (Guy *et al*., 1992). We now report that FAS and *neu* were both highly expressed in the mammary epithelium of the *neu*-N female mouse. Inhibition of FAS *in vivo* using C75, an inhibitor of FAS (Kuhajda *et al*., 2000), significantly delayed the development of cancer in this model, with four animals never developing overt carcinoma. Analysis of the mammary tissue showed a delay in mammary maturation restricted to the C75-treated *neu*-N mice; C75-treated FVB/N controls were unaffected. Concordant with the maturation delay, FAS inhibition caused a reduction in the expression of FAS, *neu*, and key elements of *neu* signal transduction including Akt, phospho-Akt, and p21. As a result, proliferation in the mammary epithelium was reduced, and apoptosis was increased. In addition, we treated a similar group of animals with C247 (4-hydroxy-5-methyl-5-octyl-5-*H*-thiophen-2-one), an FAS inhibitor chemically distinct from C75 that does not induce weight loss (McFadden *et al*., 2002; McFadden *et al*., 2004), which led to a similar delay in mammary maturation. These findings identify FAS inhibition as a potential therapeutic strategy for breast cancer chemoprevention.

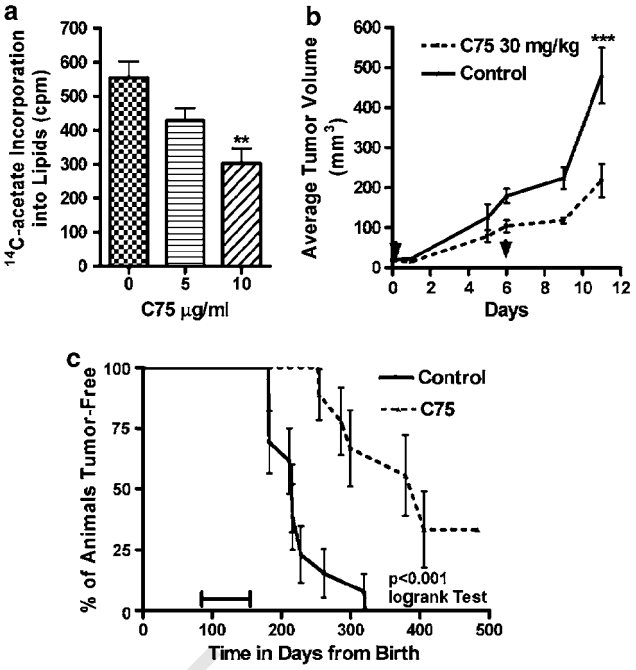

Figure 1 C75 inhibited fatty acid synthesis and growth of established cancer cell lines from *neu*-N transgenic mice, and *in vivo* mammary cancer development. (**a**) C75 inhibited fatty acid synthesis ($P=0.00$, two-tailed *t*-test) and cell growth in the NT-5 cell line. (**b**) C75 treatment (dashed line) significantly inhibited the growth of NT-2 transgenic *neu* mammary carcinoma-transplanta-ble tumors compared to vehicle controls (solid line) ($n=3$ per group, $P<0.0001$, two-way ANOVA) (*$P<0.05$; **$P<0.01$; ***$P<0.001$). (**c**) C75 was administered weekly at 30 mg/kg i.p. beginning at week 10 for 10 weeks (black bar). C75-treated animals exhibited a significant delay in mammary cancer development (dashed line) compared to vehicle controls (solid line) ($P<0.0001$, log-rank test). Four C75-treated animals did not develop cancer (*$P<0.05$; **$P<0.01$; ***$P<0.001$)

Results

Transformed cell lines derived from neu-*N mice mammary tumors undergo fatty acid synthesis and are growth inhibited by C75* in vivo

Prior to C75 treatment of *neu*-N mice, we chose to test transformed cell lines derived from the mammary tumors for evidence of fatty acid synthesis and C75 growth inhibition both *in vitro* and *in vivo*. Figure 1a shows that the NT-5 line undergoes *de novo* fatty acid synthesis. Compared to previous experiments, the rate of pathway activity in NT5 cells was about 30–50% the level of MCF-7 human breast cancer cells (Pizer *et al*., 2000). C75 at 10 μg/ml significantly inhibited fatty acid synthesis ($P=0.018$, two-tailed *t*-test). For evidence of antitumor activity of C75 *in vivo*, we chose NT2 cells because of their ability to readily form transplantable tumors (Figure 1b). C75 at 30 mg/kg significantly inhibited tumor growth ($P<0.0001$, two-way ANOVA test) compared to vehicle controls. Reversible weight loss was the only toxicity noted. C75-treated animals lost on average from 10.5 to 13.7% of their body mass within 24 h of each treatment, which was recouped within 7 days. Following the 10 weekly treatments, the weights of the controls and C75-treated animals were similar; the weights of both the control and C75-treated animals increased during the initial 10 weeks when compared to the beginning of the experiment (24.7% for control and 30.8% for the C75-treated animals). No tumor growth inhibition was noted with 15 mg/kg C75 treatment (data not shown). Since established tumor cell lines from *neu*-N transgenic mice were growth inhibited by C75 *in vitro* and *in vivo*, we proceeded to treat the transgenic mice with C75 to test if FAS inhibition would prevent or delay the development of carcinoma.

C75 treatment prevents mammary cancer development in neu-*N transgenic mice*

Derived from the FVB/N strain, *neu*-N transgenic mice express the nontransforming rat *neu* cDNA under the control of a mammary-specific promoter (Guy *et al*., 1992). As a consequence, the mice develop spontaneous mammary adenocarcinomas beginning at approximately 125 days, with the majority of the mice harboring

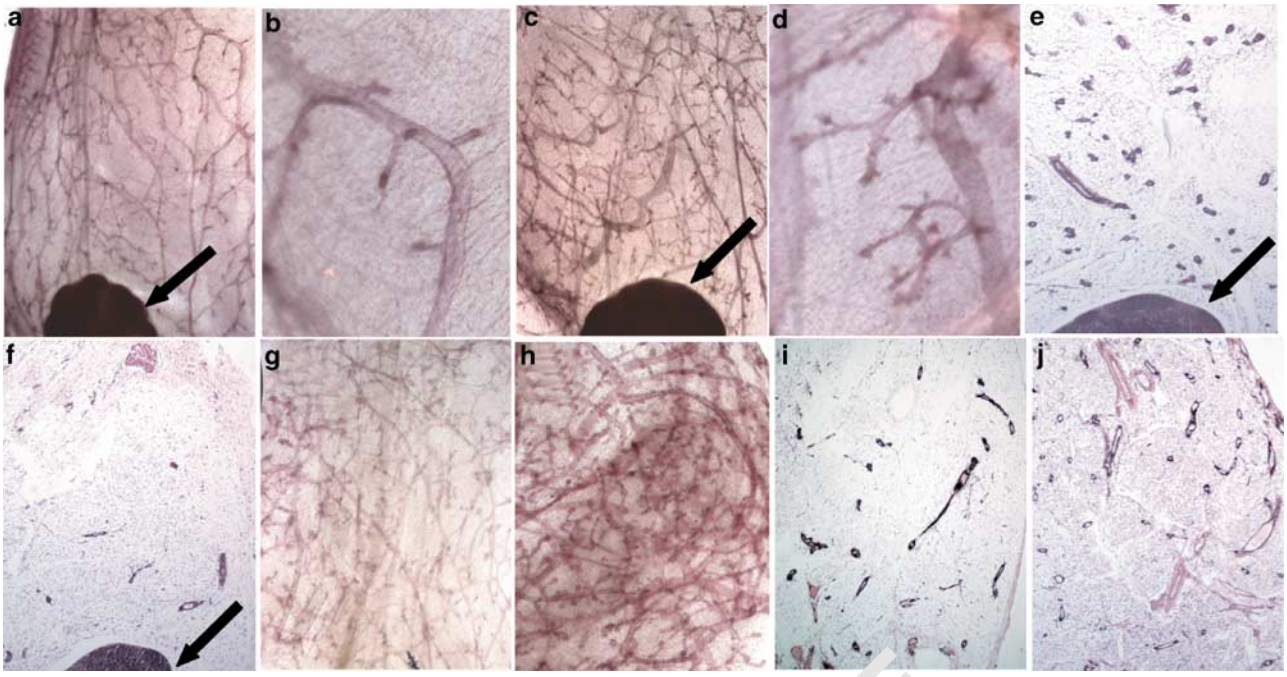

Figure 2 C75 delayed mammary gland development in *neu*-N transgenic mice but not in FVB/N controls. Whole-mount preparation of C75-treated mammary tissue (**a**) exhibits significant reduction in the number and caliber of ducts as well as a decreased number of budding epithelial structures (× 25). An enlarged (× 100) image of the area is shown in (**b**). The vehicle control whole-mount preparation (**c**) demonstrates normal number, caliber, and budding of duct structures (× 25). An enlarged (× 100) image of the area is shown in (**d**). Similar changes are reflected in histologic sections of mammary tissue from vehicle control (**e**) and C75-treated mice (**f**) (× 25). Black arrows in (**a**, **c**, **e**, **f**) denote intramammary lymph nodes, indicating similar image capture areas in all specimens. Whole-mount preparations (× 25) of mammary tissue from FVB/N vehicle controls (**g**) and C75-treated animals (**h**) showed no significant morphological differences in mammary structures. No significant alterations in histology (× 25) were noted between the mammary tissue from control (**i**) and C75-treated animals (**j**)

tumors by 300 days. This rodent model more closely resembles human breast cancer where *neu* is over-expressed, not mutated (Lofts and Gullick, 1992).

Figure 1c is a Kaplan–Meier plot of vehicle control and C75-treated transgenic mice where time until visual tumor development was scored as an event. C75 treatment significantly delayed ($P < 0.0001$, log-rank statistic) the development of mammary cancer in the *neu*-N mice. By 220 days, only 20% of the C75-treated animals developed mammary carcinoma compared to 50% in the control group. The median time without tumor for C75-treated mice was 406 days compared to 215 days for controls. Four mice in the C75 treatment group never developed mammary carcinoma. These findings demonstrate that C75 has the ability to both prevent and abrogate tumor development in *neu*-N transgenic mice.

C75 treatment retards mammary development in neu-*N transgenic mice*

To determine how inhibition of fatty acid synthesis by C75 inhibits mammary tumor development, we began by examining mammary tissue from C75-treated and vehicle control animals beginning at treatment week 2 using whole mounts and histological sections. The first significant morphological change in the C75-treated *neu*-N animals was easily identified after 8 weeks of treatment (data not shown). After 10 weeks of treatment (age 18–20 weeks), there was a significant reduction in duct development as noted by mammary whole mounts and tissue sections depicted in Figure 2. In whole mounts from C75-treated animals (Figure 2a and b), there is a significant reduction of the number of ducts, their thickness, and the number of budding epithelial structures as compared to controls (Figure 2c and d). Of particular interest is the lack of budding on the C75-treated mice compared to the arborization seen on the controls. The changes observed in the whole-mount sections were also reflected in the histologic sections of the mammary tissue. In the C75-treated animals (Figure 2f), the ducts were sparse, the epithelium thinner, and there were fewer budding epithelial structures. In the control sections, an increased number of total ducts and as well as budding ducts were evident (Figure 2e). Importantly, an intramammary lymph node was present in every mammary gland sample, which acted as a convenient internal landmark for assessing mammary development.

We also treated wild-type FVB/N mice with the same C75 dosing schedule to determine if the morphological alterations required the *neu* transgene. After 10 weeks of C75 treatment, there were no observable differences in mammary development in either the whole-mount preparations or histological sections of C75-treated (Figure 2h and j) or vehicle-treated FVB/N mice (Figure

2g and i). Additionally, there was no histological evidence of a reduction or alteration of other ductal structures in the C75-treated animals when compared to control animals, such as bile ducts, kidney tubules, or skin adnexal structures (data not shown). These data demonstrate that the morphological alterations by C75 were dependent on *neu* expression.

C75 reduces the proliferation and expression of FAS, neu*, and related genes in mammary epithelium*

To assess cell proliferation and the expression of proteins related to FAS and *neu* activity, we used immunohistochemistry performed on histological tissue sections. Since the bulk of the mammary tissue is fat and supporting stroma, we chose this method to prevent contamination by nonepithelial cells. Both FAS and *neu* were highly expressed in the ductal epithelium of the vehicle control transgenic mice (Figure 3a and c) compared to C75-treated mice (Figure 3b and d) and the wild-type FVB/N mice (data not shown).

Quantitative immunohistochemical analyses for FAS and *neu* in Figure 3e are consistent with the images in Figure 3a–d. FAS expression was markedly reduced by C75 in the transgenic animals (mean = 11 positive cells/500 total cells) compared to vehicle controls (mean = 477 positive cells/500 total cells; $P = 0.0003$) and the FVB/N control (data not shown). FAS expression in the adipose tissue surrounding the breast ducts was similar in both the transgenic animals and the wild-type controls. Immunohistochemical staining for *neu* was also decreased in the C75-treated mammary epithelium (mean = 333 positive cells/500 total cells) when compared to control animals (mean = 468 positive cells/500 total cells; $P = 0.0037$) and the FVB/N control (data not shown).

We also studied the expression of $p21^{waf1}$, Akt, and phospho-Akt (pAkt), key members of the *neu* signal transduction pathway, by immunohistochemical staining. Akt, pAkt, and $p21^{waf1}$ were all markedly decreased in mammary epithelium by C75 treatment compared to control animals. Staining for p21 was rare and weak in C75-treated animals (mean = 5 positive cells/500 total cells), with moderate intensity staining in the control group (mean = 359 positive cells/500 total cells; $P = 0.0025$). Likewise, Akt staining was rare and weak in C75-treated animals (mean = 103 positive cells/500 total cells) compared to diffuse, strong staining in the control group (mean = 473 positive cells/500 total cells; $P = 0.0018$). C75-treated mammary structures exhibited weak staining for phospho-Akt (mean = 229 cells/500 total cells), with diffuse, moderate staining present in the control group (mean = 378 positive cells/500 total cells; $P = 0.0117$). These data suggest that C75 treatment downregulates the entire *neu* signal transduction pathway in the mammary epithelium of the transgenic mice.

The effect of C75 on mammary epithelial cell proliferation was measured using immunohistochemical localization of BrdU incorporation. Anti-BrdU immunohistochemistry demonstrated typical nuclear localization. There was significantly reduced BrdU incorporation in the C75-treated animal mammary ductal structures (mean = 23 positive cells/500 total cells) as compared to control animals (mean = 159 positive cells/500 total cells; $P = 0.0086$). As an internal control, BrdU labeling was assessed in intramammary lymph nodes present in each specimen. No significant difference in BrdU labeling was appreciated between treated and control intramammary lymph nodes (data not shown). In addition to cell proliferation, we also measured apoptosis using *in situ* oligoligation in the

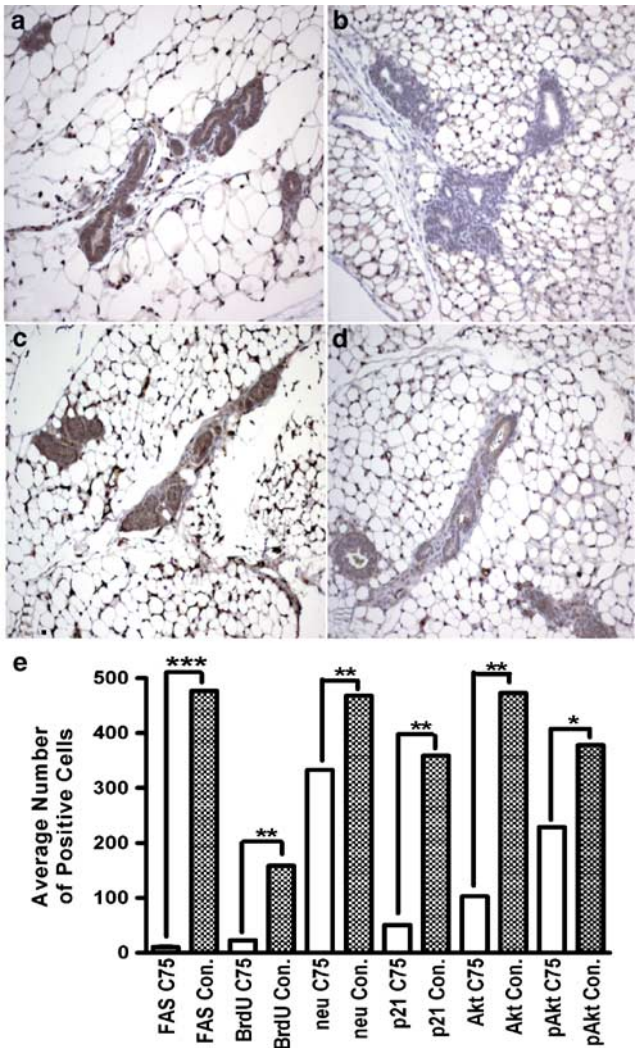

Figure 3 Immunohistochemical staining for FAS and neu, and quantitation of immunohistochemical staining for FAS, neu, Akt, pAkt, p21, and BrdU in C75-treated *neu*-N transgenic mice and vehicle controls. In the vehicle control *neu*-N animals, high levels of FAS expression were present in both ducts and adipose tissue with strong, diffuse staining (**a**). C75 treatment led to lower FAS expression in mammary ducts with weak and focal staining (**b**). *neu* expression was increased in the *neu*-N mammary tissue (**c**) compared to C75-treated animals (**d**). (**e**) FAS and *neu* expression in the C75-treated mammary tissue was markedly reduced compared to the vehicle controls ($P = 0.0003$ and 0.0037, respectively). Additionally, expressions of p21, Akt, and pAkt were all decreased in C75-treated animals compared to control animals ($P = 0.0025$, 0.0018, and 0.0117, respectively) along with BrdU incorporation ($P = 0.0086$) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) (all images at $\times 200$)

histologic sections. Apoptosis was present in the C75-treated animals (mean = 3.3 positive cells/500 cells) whereas no apoptosis was present in the control group (data not shown). Taken together, these data demonstrate that C75 downregulates FAS and *neu* expression along with key molecules in the *neu* signal transduction pathway. In addition, cell proliferation is reduced and apoptosis is increased leading to drastically reduced mammary development in the *neu* transgenic animals. Importantly, all of these effects were restricted to *neu* overexpressing mammary epithelial cells.

C247 treatment retards mammary development in neu-*N transgenic mice*

Similar to C75, C247 also retarded mammary development in the *neu*-N transgenic mice. After 10 weeks of treatment (age 18–20 weeks), there was a significant reduction in duct and lobular development as noted by mammary whole mounts and tissue sections depicted in Figure 4. Whole mounts of the vehicle-treated mice depict extensive lobular development (Figure 4a and b) compared to C247-treated animals (Figure 4c and d). Similarly, histological sections of the control mice show lobular hyperplasia with mitotic figures and nuclear debris suggestive of apoptosis (Figure 4e and f). In contrast, C247 treatment dramatically reduced both duct and lobular development (Figure 4g and h). C247 is a thiolactomycin derivative chemically distinct from C75 (Figure 4i).

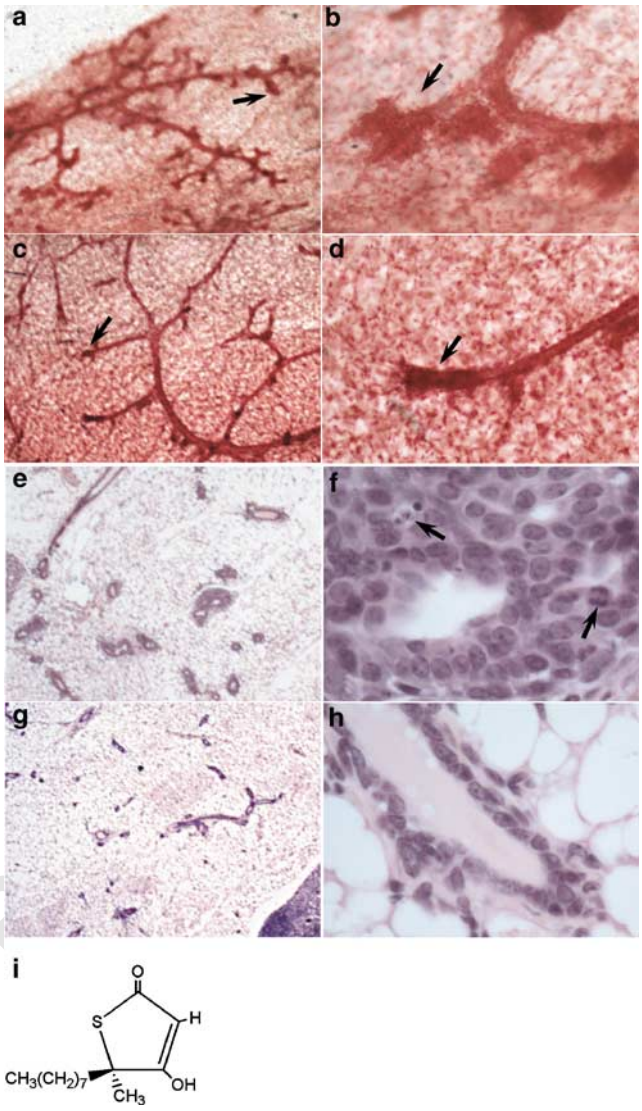

Figure 4 C247 delayed mammary gland development in *neu*-N transgenic mice. Whole-mount preparation of vehicle control mammary tissue (**a**) demonstrates normal number, caliber, and budding of duct structures (arrow) (× 25). An enlarged (× 100) image of the area is shown in (**b**) with the arrow denoting a mammary lobule. C247 treatment (**c**) caused significant reduction in the number and caliber of ducts as well as a decreased number of budding epithelial structures (arrow) (× 25). An enlarged (× 100) image is shown in (**d**); the arrow marks a mammary lobule. The histologic section of mammary tissue from a vehicle control shows significant lobular hyperplasia (× 25) (**e**) with arrows showing a mitotic figure and nuclear fragmentation suggestive of apoptosis (**f**) (× 400). C247 treatment reduced the total number and caliber of ducts (× 25) (**g**) with an absence of lobular hyperplasia (**h**). The chemical structure of C247 is shown (**i**)

Discussion

C75 treatment of *neu*-N transgenic mice led to a significant delay in mammary tumor development and, in some animals, complete prevention of the disease. The chemopreventive effect of C75 was manifested by the selective inhibition of both mammary duct and lobule development restricted to the *neu*-N transgenic mice. Importantly, C75 had no effect on mammary development in wild-type FVB/N mice. Concomitant with the morphological changes in the mammary tissue, C75 treatment downregulated FAS and *neu* expression, and other key molecules in the *neu* signal transduction pathway in the mammary epithelium. As most pre-invasive breast cancer and premalignant breast lesions overexpress *neu*, these data provide compelling evidence that FAS is a potential drug target for chemoprevention.

Several *in vitro* studies have shown a link between *neu* expression and fatty acid synthesis in breast epithelial cells. In model system using human breast epithelial cells transfected with *neu*, Kumar-Sinha *et al*. (2003) demonstrated elevated FAS expression, which was driven by *neu* through PI3K/Akt signaling, via a direct effect on the *cis*-acting elements in the FAS promoter. Additionally, their results demonstrated that pharmacological inhibitors of FAS (including C75) preferentially killed HER2/*neu* overexpressing breast epithelial cells relative to matched vector controls. Recently, Menendez *et al*. (2004) observed that $p185^{HER2}$ was downregulated by pharmacological FAS inhibition, suggesting a link between FAS and carcinogenesis through its regulation of oncogenes. Others have also found an association between Akt, FAS, and human breast (Yang *et al*., 2002) and ovarian (Wang *et al*., 2003) cancer. Utilizing three different ovarian carcinoma cell lines, Wang *et al*. demonstrated that inhibition of FAS activity by C75 resulted in downregulation of phosphorylated (active) Akt, which preceded the induc-

tion of apoptosis. Collectively, their data demonstrated that ovarian cancer cells with constitutively active Akt were protected from apoptosis, and that inhibition of the PI3K/Akt pathway increased their sensitivity to C75- and cerulenin- (another FAS inhibitor) induced apoptosis, at least partially through Akt-regulated downregulation of FAS. Taken together, the downregulation of pAKT and $p185^{HER2}$ by C75 inhibition of FAS *in vitro* is similar to our findings *in vivo* and provides a mechanism whereby pAKT, FAS, HER2/*neu,* and p21 are all downregulated in the mammary epithelial cells of C75-treated *neu*-N mice. These data suggest that FAS and fatty acid synthesis may play a role in oncogenesis through regulation of key oncogenic pathways on which cancer cells depend for survival (Weinstein, 2002) and support FAS inhibition as a means to selectively kill breast epithelial cells harboring *neu* overexpression.

We chose to initiate the treatment of the transgenic mice at the same time and dose regimen as was used in our MCF7 xenograft studies. The length of treatment was chosen to end at about the midway point (140 days) for the average appearance of tumors (~300 days). The time of onset for C75 treatment was chosen based upon our experience that young adult female mice tolerate C75 treatment without evidence of overt toxicity. Future studies will address the requirement for timing the onset and duration of therapy to maximize the reduction of tumor development. In patients, it would not be desirable to treat during the period of breast development as this could adversely affect cosmesis and lactation. However, since preneoplastic lesions do not usually appear during adolescence, treatment would likely be able to be postponed until well into adulthood.

Morphological analysis of the mammary tissue after 10 weeks of C75 treatment of *neu*-N and wild-type FVB/N mice demonstrated a striking delay in mammary development that required the presence of the *neu* transgene. The ducts in the treated *neu*-N mice had a reduced caliber with an absence of arborization that was seen in the *neu*-N vehicle control and C75-treated FVB/N mice. Importantly, C75 treatment did not result in morphological changes in other duct structures in other organs such as the skin, liver, kidney, or pancreas, for example. Moreover, we have not identified an effect of C75 treatment on proliferating cell compartments in the skin, gastrointestinal tract, or bone marrow. Importantly, FAS expression in mammary adipose tissue was similar in the transgenic animals and the wild-type controls. FAS expression in adipose tissue is not under the control of the *neu* gene, but is likely under the control of SREBP-1 that would not be affected by the *neu* transgene (Boizard *et al*., 1998; Kim *et al*., 1998). While reversible weight loss constitutes the dose-limiting toxicity of C75 (Loftus *et al*., 2000; Pizer *et al*., 2000), the altered morphology was restricted to breast epithelial cells harboring the *neu* transgene that were exposed to C75.

Given the increasing evidence for the intertwining roles of *neu*, FAS, and PI3K/Akt signaling in cell growth and apoptosis, we sought to determine if C75 exerts these morphological changes through altered expression of these key proteins. As anticipated, the *neu*-N mice had increased expression of both *neu* and FAS compared to the wild-type FVB/N mice. C75 treatment of the transgenic mice, however, reduced expression of both *neu* and FAS to levels below that seen in the wild-type controls. C75 also reduced the expression of both Akt and pAkt along with p21, which are important in the cascade of PI3K signaling. The mechanism responsible for the antiapoptotic effect of *neu* overexpression occurs through the AKT pathway leading to AKT phosphorylation and cytoplasmic localization of p21/Waf1 (Zhou *et al*., 2000). This promotes the development of preneoplastic lesions such as atypical hyperplasia and *in situ* carcinoma, between 22 and 30 weeks of age in *neu*-N mice (Boggio *et al*., 2000). Based on these findings, we measured the effect of C75 on cell proliferation and apoptosis in this model. C75 significantly reduced BrdU incorporation indicating reduced cell proliferation, and increased apoptosis. Therefore, we hypothesize that C75 treatment impedes mammary development in *neu*-N mice at least in part through the downregulation of the PI3K/Akt pathway enhancing apoptosis of *neu* expressing cells. In addition to potentially killing *neu* overexpressing breast epithelial cells, the reduction of mammary development seen in the C75-treated *neu*-N mice may also, in part, be responsible for its chemopreventive effect.

Importantly, C247, an FAS inhibitor chemically distinct from C75, also impedes mammary development in the *neu*-N mice. Since C247 does not stimulate carnitine palmitoyltransferase-1 activity and increase fatty acid oxidation like C75 (Thupari *et al*., 2002, 2004), C247-treated mice do not experience significant weight loss compared to vehicle-treated controls. Thus, the chemopreventive effects of C247 and C75 are attributable to their common functionality, FAS inhibition, not to increased fatty acid oxidation or weight loss.

The effect of C75 on FAS expression has also been explored in a recent study of the transgenic adenocarcinoma of the prostate (TRAMP) model of prostate cancer (Pflug *et al*., 2003). Upregulation of FAS expression and activity in the transgenic mouse prostates were evident in prostate epithelia as early as 12 weeks of age in prostatic intraepithelial neoplasia (PIN) lesions, and further increased with age and tumor progression, culminating in metastatic lesions to the liver, kidney, lymph nodes, and lung. All other non-neoplastic tissues demonstrated low FAS levels equivalent to nontransgenic littermates, indicating prostate-specific upregulation of the enzyme with tumor progression. FAS pathway inhibition by C75 and cerulenin in cell lines and tissues resulted in a dose-dependent reduction in cell survival and decreased enzyme activity. These findings are similar to those we have demonstrated in our transgenic mouse model of breast cancer, and support the hypothesis that the upregulation of FAS expression may play a significant role in prostate tumorigenesis, and as in breast cancer, FAS may serve as a therapeutic target.

These findings have direct bearing on human breast cancer prevention. A recent study of *neu* expression in human breast cancer precursor lesions found amplification of *neu* in 21/22 cases of *in situ* duct carcinoma (Xu *et al*., 2002). Additionally, *neu* overexpression was discovered in 7/13 cases of atypical duct hyperplasia, an early, potentially premalignant breast lesion. Ordinary duct hyperplasia without atypia and normal breast ducts did not overexpress *neu*. Thus, the only structures in the human breast that overexpress *neu* are either premalignant atypical duct proliferations, *in situ* cancer, or infiltrating breast cancers. FAS thus provides a potential novel target for the treatment of women at high risk for the development of breast cancer since FAS inhibition should only affect premalignant or malignant cells without altering normal breast structures or other non-neoplastic tissues.

Materials and methods

Mice

neu-N transgenic mice (Guy *et al*., 1992), bred to homozygosity as verified by Southern blot analysis, were obtained from Dr Elizabeth Jaffee at the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins. FVB/N mice were obtained commercially from the Jackson Laboratory. During all studies, mice were maintained on ordinary Purina mouse chow and water *ad lib*. All experiments involving the use of mice were performed in accordance with protocols approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine (Baltimore, MD).

Cell lines, chemicals, and fatty acid synthesis

NT-5 and NT-2 cell lines derived from spontaneous breast adenocarcinomas from *neu* transgenic mice were developed by Dr Elizabeth Jaffe (Department of Oncology, Johns Hopkins Medical Institutions) (Reilly *et al*., 2000). Cells were cultured in RPMI (Life Technologies Inc. Grand Island, NY, USA) with 20% fetal bovine serum at 37°C in 5% CO_2. The FAS inhibitors C75 and C247 were obtained from FASgen Inc. (Baltimore, MD, USA). C75, dissolved in DMSO, was added from 5 mg/ml stock solutions; the final DMSO concentration in cultures was $\leqslant 0.2\%$. Fatty acid synthesis was measured by ^{14}C acetate incorporation into total lipids in NT5 cells (Pizer *et al*., 1996).

C75 treatment of NT2-transplantable tumors

Prior to the C75 treatment of the *neu*-N transgenic mice, we treated NT-2 transplantable tumors with C75 to determine if the *neu*-transformed mouse mammary epithelial cell line was sensitive to fatty acid synthesis inhibition *in vivo*. To establish the NT-2 tumor-transplantable tumors, 10^6 NT-2 cells were injected into the flank of six FVB/N mice. When tumors became measurable in three dimensions with calipers, three mice were treated with C75, diluted in RPMI culture medium, intraperitoneally (i.p.) at 30 mg/kg on days 0 and 6. In a similar experiment, three mice were treated with C75 at a concentration of 15 mg/kg on the same dosing schedule.

C75 prevention of spontaneous tumor development in neu-*N transgenic mice*

To test the effect of C75 on tumor development in *neu*-N transgenic mice, 15 animals were treated with C75 or vehicle once a week (30 mg/kg in RPMI, i.p.) beginning at 12 weeks of age and continuing for 10 weeks. Since *neu*-N mice develop preneoplastic lesions at about 22 weeks (Boggio *et al*., 2000), the C75 treatment preceded the appearance of these lesions. Animals were monitored visually for tumor development and the time of tumor development was recorded. Data collection continued for a total of 483 days.

Serial morphological and immunohistochemical analysis of C75- or C247-treated neu-*N transgenic mice*

A total of 15 (8- to 10-week-old) *neu*-N transgenic mice were treated i.p. with C75 (25 mg/kg) weekly, along with 15 vehicle controls (RPMI). Three mice from the treatment and control groups were killed by carbon dioxide asphyxiation at 2-week intervals beginning at treatment week 2 (2 weeks after the first C75 treatment at 8–10 weeks of age). All animals received 1 mg of BrdU 2 h prior to killing. Entire inguinal mammary glands were removed. Tissues were fixed in formalin for histology and Carnoy's fixative for whole-mount preparation (Mueller *et al*., 2002). Additionally, kidney, liver, and skin samples were collected for histology. Mammary tissues from age-matched FVB/N control mice were removed for similar analysis at treatment week 10 (age 18–20 weeks).

Immunohistochemistry was performed with the following antibodies: FAS (rabbit polyclonal anti-FAS antibody, FASgen Inc., Baltimore, MD, USA), BrdU and p21/Waf-1 (Dako, Carpinteria, CA, USA), Akt and phospho-Akt (Cell Signaling Technology, Beverly, MA, USA), and *neu* (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), apoptosis (ApopTag Peroxidase *In Situ* Oligo Ligation Kit, Serologicals Corporation, Temecula, CA, USA). Staining was assessed by counting the number of positive cells per 500 total cells in the ductal and lobular structures at $\times 400$. *neu*-N transgenic mice were also treated with C247 (30 mg/kg) diluted in 50 μl DMSO on the same treatment schedule as C75, and mammary tissue was removed for histological and whole-mount analysis.

Statistical analysis

All data are presented as means $\pm$ standard error of the mean from multiple determinations. Data were analysed by linear regression, two-tailed unpaired *t*-tests, two-way ANOVA, or Kaplan–Meier plots with log-rank statistics where applicable using Prism 4.0 (Graph Pad Software).

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